

## Original Article

# Atorvastatin inhibits vascular smooth muscle cell phenotype transformation in cerebral hemorrhage rats by down regulating KLF-5

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**Abstract:** Objective: To investigate whether atorvastatin (ATV) could inhibit vascular smooth muscle cell (VSMCs) phenotype transformation by down regulating KLF-5 in cerebral hemorrhage (CH) rats. Methods: Cerebral hemorrhage animal model was established successfully, VSMCs were isolated by adhesion method, followed by primary cultured and identified. After 3-5 generations, cells were used for the subsequent experiment. The expressions of mature VSMCs marker proteins, including SM-actin (SMA), SM-22 $\alpha$ , SM-MHC and OPN were detected by Western blotting. The proliferation and migration of VSMCs were detected by MTT assay and wound healing method, respectively. The morphology of VSMCs was observed by detecting the expression of SMA by using cell immune fluorescence. Then KLF-5 over-expression or blank control plasmid were transfected into VSMCs *in-vitro*, phenotypic transformation of VSMCs were detected with ATV treatment by using the above-mentioned experimental methods. Results: MTT and wound healing results showed that compared with control group, VSMCs proliferation and migration were significantly reduced in ATV groups. Western blotting results showed that the expression of SMA and SM-MHC in ATV groups was increased, and the expression of OPN and KLF-5 was decreased significantly compared with control group. Cell immune fluorescence results showed that the morphology of VSMCs became thinner and longer in ATV groups when compared with control group. However, after transfection of KLF-5 over-expression plasmid, the proliferation and migration of VSMCs were significantly inhibited, cell morphology and the phenotype-associated proteins concentration were changed. Conclusion: ATV inhibited VSMCs phenotype transformation in CH rats by down regulating KLF-5 expression, which providing a new idea for gene therapy in the development of CH.

**Keywords:** Cerebral hemorrhage, atorvastatin, vascular smooth muscle cell, phenotype transformation

## Introduction

Cerebral hemorrhage (CH) accounted for 20% to 30% of cerebral vascular diseases in China and remained the leading cause of mortality largely because of their high morbidity characteristic [1]. At present, average 112 per 100,000 people suffered from the disease and it led the first place in the entire cerebral vascular disorders [2]. Intracranial vascular fibrosis, decreased vascular elasticity, reduced tensile capacity often appeared in CH patients, especially in hypertensive cerebral hemorrhage patients because of long-term persistent high blood pressure [3-5]. Under the emotional, cold and other stimulating conditions, blood vessels prone to rupture, blood flowed into the brain

and hematomas were formed within 30 minutes of bleeding. Vascular brain edema was the most important secondary pathological characteristic following cerebral hemorrhage, which was also the main reason leading to worsening of the patient's condition and neurological dysfunction [6, 7].

Proliferation and migration of vascular smooth muscle cells (VSMCs) were an important basis in the development of atherosclerosis, which was closely related to CH [8]. Recent studies have shown that VSMCs phenotypes were distinct in different differentiation stages, the physiological characteristics of VSMCs varied widely among them and different phenotypes of VSMCs can be transformed into each other

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when the external condition changed [9, 10]. Statins such as atorvastatin (ATV) could delay the development of atherosclerosis, in further to reduce the incidence of CH by lipid-lowering, improving endothelial function, reducing or eliminating inflammatory response, inhibiting vascular smooth muscle proliferation and so on [11]. In addition, ATV could significantly reduce the area of atherosclerotic plaques in the aorta of LDLR<sup>-/-</sup> mice [12]. However, whether ATV can inhibit the phenotypic transformation of VSMCs in patients with CH and its mechanism has not been proven. In this study, the effects of different concentrations of ATV on VSMCs phenotype and KLF-5 expression were observed. The effect of KLF-5 on the phenotypic transformation of VSMCs in rats with CH was also investigated, in order to explore whether KLF-5 was involved in the phenotypic transformation of ATV inhibiting VSMCs in CH rats.

### Materials and methods

#### *Experimental materials*

SPF SD rats weighing 200-220 g were purchased from the Academy of Medical Sciences Experimental Animal Center in Zhejiang Province. Experimental reagents including: fetal bovine serum (Gibco), atorvastatin (Sigma), RNA extraction reagents (Invitrogen), endotoxin-free mini/maxi plasmid extraction kit and liposome 2000 (Invitrogen, Shanghai), DAPI (Rcohe), MTT (Emresco), rabbit or mouse anti-rabbit SMA, SM-22 $\alpha$ , SM-MCH, OPN, KLF-5,  $\beta$ -actin polyclonal antibodies (Abcam), horseradish peroxidase-labeled goat anti-rabbit or anti-mouse secondary antibody, FITC, FRITC-labeled goat anti-rabbit IgG (Jackson Company), protein immune-blotting related reagents (Beyotime biotechnology research institute, Jiangsu). All animal experiments were approved by the Experimental Animal Ethics Committee of Qilu Hospital of Shandong University.

#### *Experimental methods*

**VSMCs primary cell culture:** CH model was induced by injecting collagenase into the caudate nucleus of rats, and VSMCs were isolated by adhesion method. Cellular immune fluorescence detection of SMA was used to identify VSMCs purity through the relationship between SMA and DAPI staining. Cells from passage 3-5

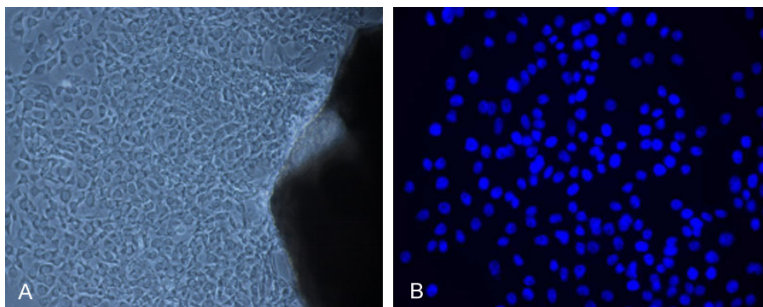
were used for subsequent experiments. Liposome 2000 was used to transfect the cells in DMEM medium without fetal bovine serum. After the transfection for 6 h, the medium was replaced with 10% fetal bovine serum and the cells were further cultured for 24 h. The total protein in the cells was extracted and the transfection efficiency was determined by Western blotting analysis of KLF-5 expression.

**Experimental groups:** The experiment composed of two parts, the first part was used to test whether ATV could inhibit the expression of KLF-5 and the phenotype of VSMCs. Experimental groups included control group and three different concentrations of ATV groups (1, 10, 100  $\mu$ mol/L). In the second part, we verified that ATV could inhibit the phenotypic transformation of VSMCs by down regulating KLF-5 expression. The cells were divided into four groups: control group, ATV group, KLF-5 group and ATV + over-expressing KLF-5 group.

**Construction of KLF-5 over-expression plasmid and cell transfection:** The sequence of KLF-5 gene was searched by Pub-med and primers were designed according to KLF-5 mRNA sequence in Gen-bank. Then the KLF-5 gene fragment was amplified by the Primestar method, the obtained KLF-5 gene fragment was digested and ligated, and the ligation product was transformed into Escherichia coli DH5 $\alpha$ . The recombinant plasmid was screened and verified them. The VSMCs were seeded in 6-well plates and liposome 2000 was transfected into KLF-5 or control group (100 pmol/group) when the VSMCs grown to 50% to 70% density. After 24 h, total protein was extracted for identification of transfection efficiency, and subsequent experiments were performed.

**Western blotting:** After 48 h of cell culture, the total proteins were extracted from each group and quantified with BCA method, and these proteins were then subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes. After non-specific binding was blocked with 5% nonfat milk at room temperature for 2 h, membranes were incubated with primary antibodies as follows: SMA (1:1000), SM-22 $\alpha$  (1:1000), SM-MHC (1:1000), OPN (1:1000), KLF-5 (1:1000) and  $\beta$ -actin (1:1000, Santa Cruz Biotechnology). Then, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 h.

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**Figure 1.** Identification of the VSMCs. A. VSMCs climbed out of the tissue about 8 days later and fused together 2 weeks later. B. VSMCs were typical “peak valley” growth pattern after passage and the purity of the cells was over 99% detected by immune fluorescence staining ( $\times 400$ ).

Immuno blots were visualized by enhanced chemi-luminescence (ECL kit, Santa Cruz Biotechnology) and recorded by quantity-one software. The relative integrated density values were calculated with  $\beta$ -actin as an internal control.

**MTT method:** VSMCs were cultured in 96-well plates according to the above-mentioned method. The corresponding intervention factors were added following synchronization and each group of cells was provided with 3 wells and 2 cells without cells. The cells were cultured for 24 h, 48 h and 72 h, respectively. After incubation, 20  $\mu$ l of MTT solution was added to each well and incubation was continued for 4 h~6 h at 37°C. The culture supernatant was discarded carefully and 150  $\mu$ l of DMSO solution was added to per well with 10 minutes oscillation. The OD of each well was measured and recorded by enzyme-linked immune sorbent assay at a wavelength of 492 nm. The growth curve of VSMCs in each group was plotted taken time as the horizontal axis and absorbance value as the vertical axis.

**Wound healing:** VSMCs were cultured in 6-well plates according to the above-mentioned method. After synchronization, cell proliferation was inhibited by 1.8 mmol/l hydroxyurea for 12 h, and cell scratches were generated by 100  $\mu$ l yellow tip vertical orifice plates, the cell culture medium was aspirated and washed three times with PBS. The cells were cultured under the corresponding culture conditions, and images were taken at 0, 12, 24, 48 and 72 h. Cell migration areas were calculated by using image pro plus 6.0, the ratio between the migration areas and the original scratch area

was used to show the migration degree of VSMCs in each group.

**Cell immune fluorescence:** VSMCs were cultured in 96-well plates according to the above-mentioned method. The corresponding intervention factors were added following synchronization. After 48 h of cell culture, the cells were washed three times with PBS, fixed with 4% paraformaldehyde, 0.25% Triton X100 was used to permeate the cell membrane, blocked at room

temperature for 1 h with goat serum, and anti-SMA antibody diluted 250 times, then overnight at 4°C, washed three times with PBST, following goat anti-rabbit secondary antibody conjugated with FITC was added and incubation was performed at room temperature for 1 h, images were taken by fluorescence microscope and data were analyzed by image pro plus 6.0 software.

### Statistical analysis

SPSS 20.0 software was used to analyze the data. The experimental data were expressed as  $x \pm s$ . One-way ANOVA was used to compare the two group data. One-way ANOVA and LSD were used to compare the multiple group data.  $P < 0.05$  was considered statistically significant.

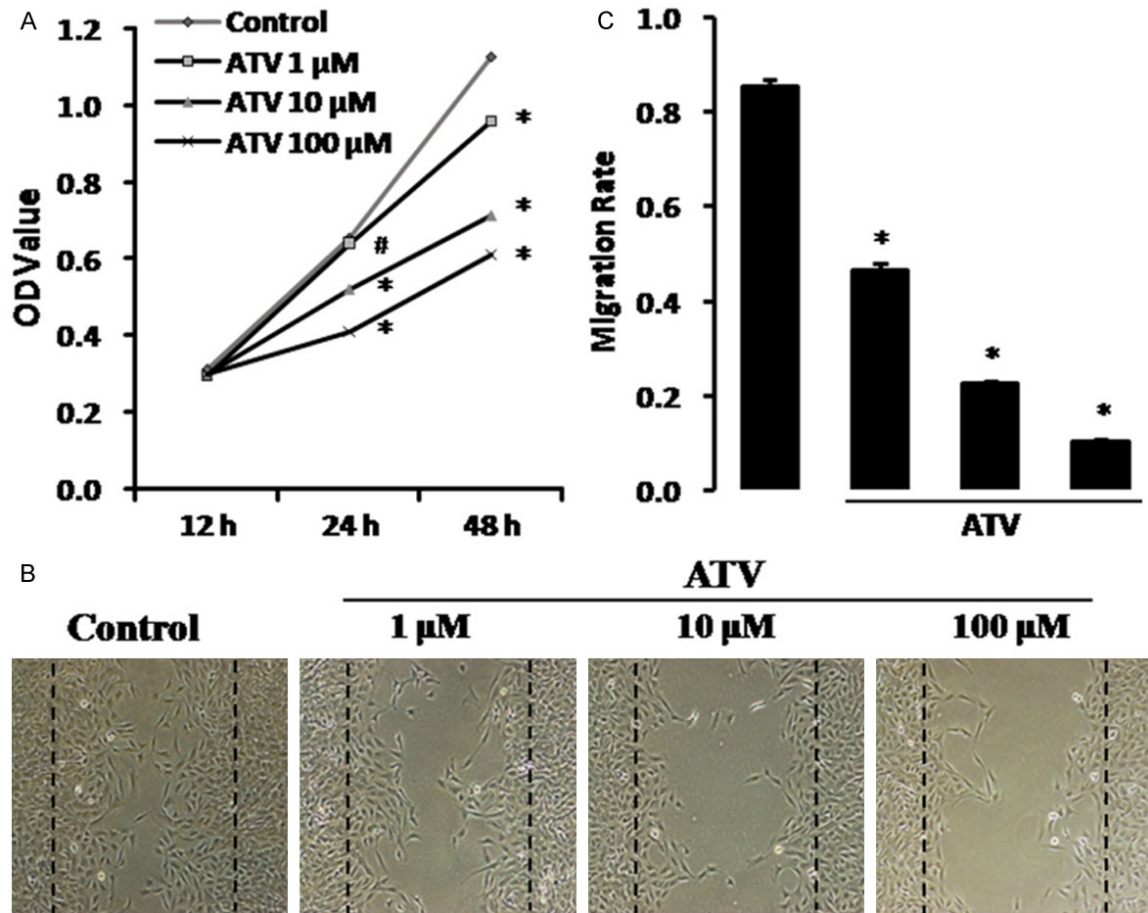
## Results

### VSMCs primary cell culture identification

Tissue culture method was used to culture the VSMCs in CH rats. Results showed that cells were formed around the tissue on the 8<sup>th</sup> day, cells were fused and can be used for passage in the 2<sup>nd</sup> week (**Figure 1A**). After passage, VSMCs were identified by cell immune fluorescence with SMA, and the cell purity was above 99% following DAPI staining (**Figure 1B**).

### ATV inhibited VSMCs phenotype transformation

**ATV inhibited VSMCs proliferation and migration:** After treatment with intervention factors for 12 h, no significant differences between



**Figure 2.** ATV inhibited VSMCs proliferation (A) and migration (B and C). #P<0.05, \*P<0.01 compared with control group. ATV: Atorvastatin.

the OD values were observed among different groups. Compared with control group, the OD values reduced significantly with 24 h treatment in a dose-dependent manner. The result showed that ATV could inhibit VSMCs proliferation (Figure 2A). In addition, after treatment with intervention factors for 48 h, migration degree obviously decreased in ATV groups when compared with control group, also along with a concentration-dependent manner, which indicating that ATV could inhibit VSMCs migration (Figure 2B and 2C).

**ATV inhibited VSMCs morphological changes:** The fluorescence patterns of SMA in VSMCs among different groups were different. As shown in Figure 3, the cell morphology became round and SMA arrangement disorder in control group. However, the VSMCs were elongated and fusiform, and SMA was well arranged in ATV groups when compared with control group.

**ATV affected the expression of cell phenotype-associated protein and KLF-5 in VSMCs:** There was no significant difference in the expression of SMA in VSMCs among experimental groups. Moreover, when compared with control group, SM-22α and SM-MHC expression in VSMCs increased significantly, OPN and KLF-5 expression reduced significantly in ATV groups, accompanied by a dose-dependent manner (Figure 4A and 4B).

**Over-expression of KLF-5 reversed the inhibition effect of ATV on VSMCs phenotype transformation**

**Over-expression of KLF-5 reversed the inhibition effect of ATV on VSMCs proliferation and migration:** Compared with control group, the OD values and migration values were obviously reduced in ATV groups without cell transfection. However, after transfection of the KLF-5



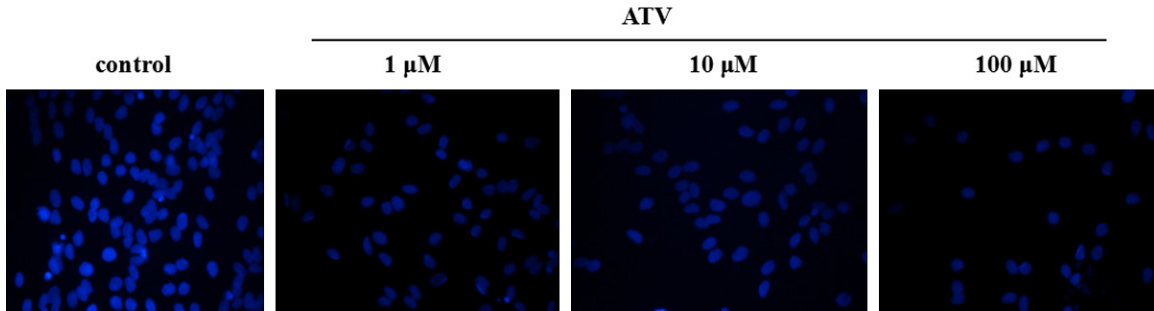


Figure 3. ATV inhibited VSMCs' morphology changing.

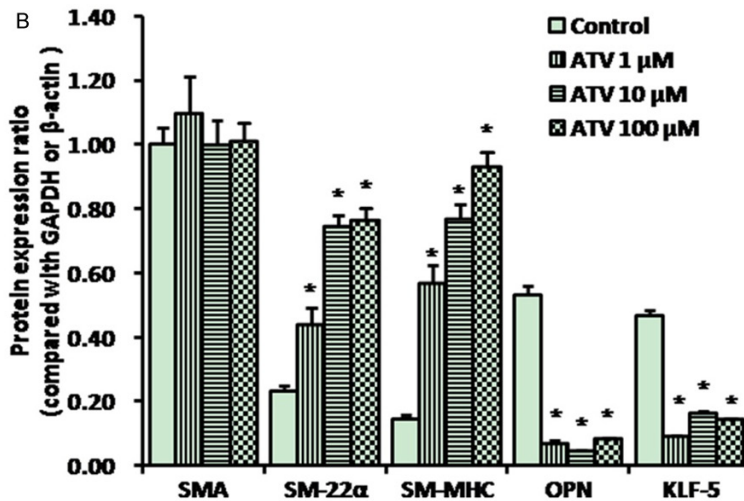
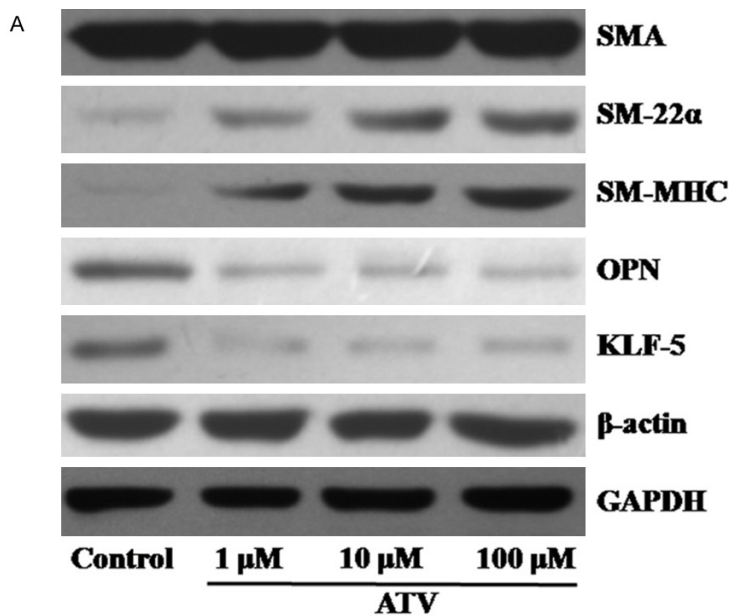


Figure 4. Effects of different concentrations of ATV on the expression of SMA, SM-22α, SM-MHC, OPN and KLF-5. The data were expressed as Mean ± SD, N=5, \*P<0.01, compared with control group.

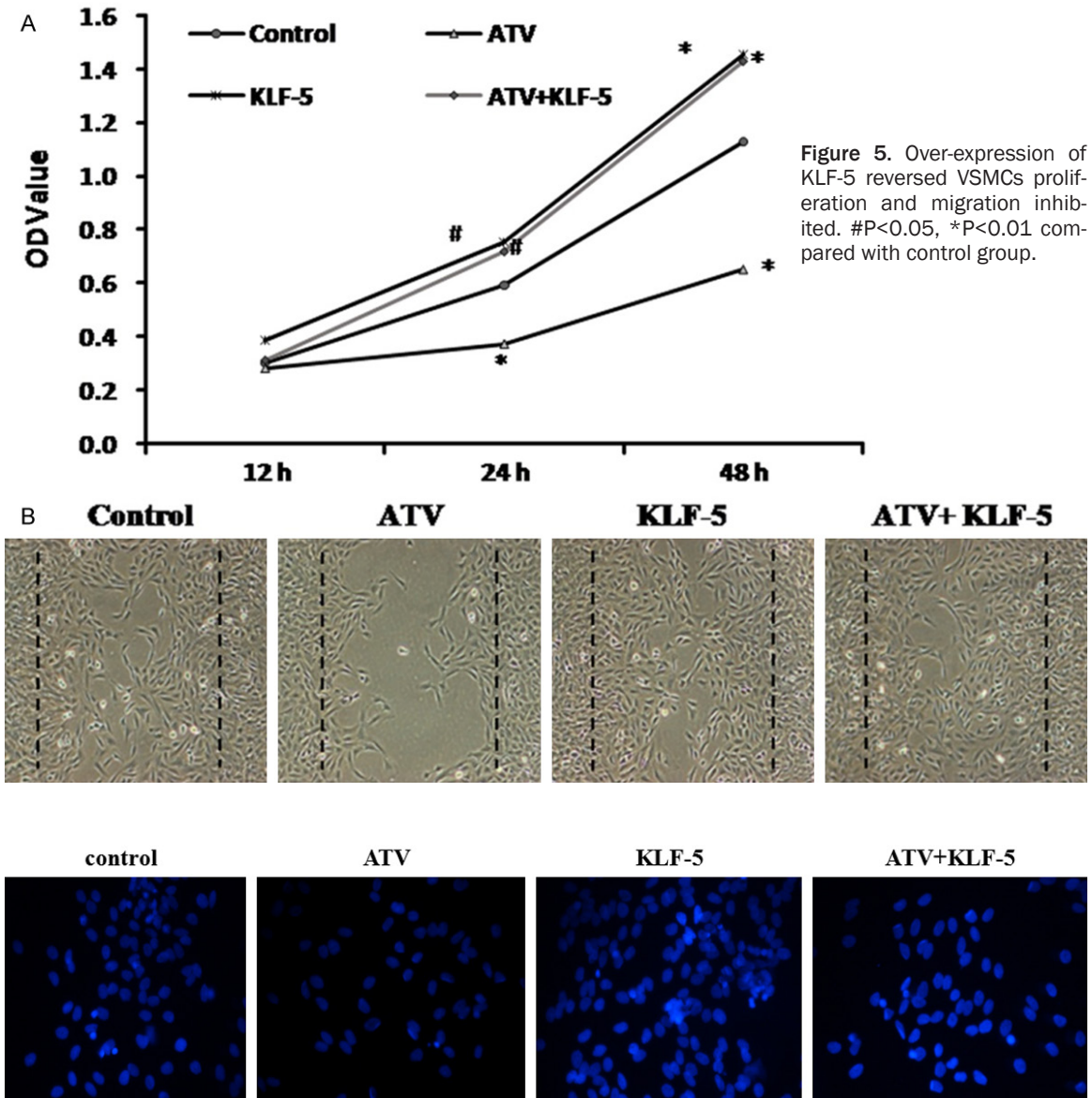
plasmid, the OD values and migration were significantly increased in KLF-5 group and ATV

+ KLF-5 group compared with control group, which indicating that over-expression of KLF-5 could improve the proliferation and migration of VSMCs and reverse the inhibition effect of ATV on VSMC proliferation and migration (Figure 5A and 5B).

Over-expression of KLF-5 reversed the inhibition effect of ATV on VSMCs morphological changes: VSMCs were elongated and fusiform, and the SMA was well arranged in ATV groups when compared with control group. After transfection of the KLF-5 plasmid, the morphology of VSMCs became rounded and the SMA was disordered in KLF-5 and ATV + KLF-5 groups. The result showed that over-expression of KLF-5 could lead to the round shape of VSMCs, reversing the inhibition effect of ATV on morphological changes (Figure 6).

Over-expression of KLF-5 reversed the inhibition effect of ATV on VSMCs phenotype-associated protein and KLF-5: Compared with control group, SM-22α and SM-MHC expression in VSMCs increased significantly, OPN and KLF-5 expression reduced significantly in ATV groups. After transfection of the KLF-5 plasmid, SM-22α and SM-MHC expression in VSMCs reduced obviously, OPN expression increased

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**Figure 5.** Over-expression of KLF-5 reversed VSMCs proliferation and migration inhibited. #P<0.05, \*P<0.01 compared with control group.

**Figure 6.** Over-expression of KLF-5 reversed VSMCs morphological changing.

significantly in ATV groups, which demonstrating that over-expression of KLF-5 could reverse the inhibition effect of ATV on VSMCs phenotype-associated protein and KLF-5 (Figure 7A and 7B).

### Discussion

VSMCs were well-differentiated and showed spindle cell morphology, possessing the function of maintaining blood vessel shape and contracting blood vessel in normal intracranial intravascular [13]. The main characteristics of VSMCs were low proliferative capacity, slow

migration, less protein secretion and others [9]. However, when CH or other vascular diseases occurred, VSMCs were dedifferentiated and transformed into undifferentiated VSMCs, with round cell morphology and reduced contractile function, showing enhanced proliferation ability, increased migration and protein secretion capability [14-16], which played an important role in the development of CH.

VSMCs were first found with different cell phenotypes, now many proteins with high specificity can be regarded as markers of mature VSMCs, such as SMA, SM-MHC, calponin, SM22 $\alpha$ ,

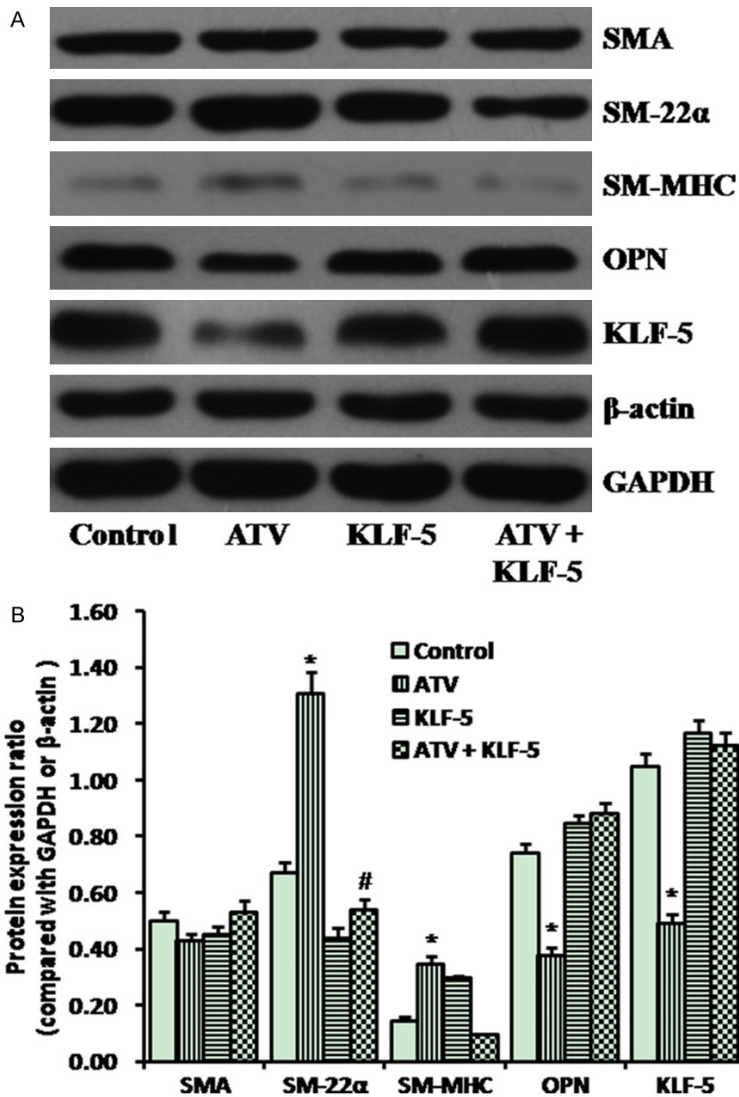


Figure 7. Over-expression of KLF-5 reversed VSMCs phenotype-associated protein and KLF-5. The data were expressed as Mean  $\pm$  SD, N=5, #P<0.05, \*P<0.01 compared with control group.

smoothelin and other proteins involved in cytoskeleton formation including h-caldesmon, telokin,  $\beta$ -vinculin, metavinculin, desmin [17-20]. These above proteins were highly expressed in differentiated VSMCs, and their expression decreased with the dedifferentiation degree of VSMCs. However, the expression level of OPN, MMPs, and sugar matrix proteins was positively correlated with the dedifferentiation [21], which was similar to our findings. Therefore, the detection of VSMCs proliferation and migration capacity, along with the expression of SMA, SM-MHC, SM22 $\alpha$ , OPN protein was applied to identify different cell phenotypes of VSMCs.

ATV was a selective inhibitor of HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase), which reduced plasma cholesterol and lipoprotein levels by lowering cholesterol in the liver biosynthesis, while increasing LDL uptake and catabolism by increasing the number of low-density lipoprotein (LDL) receptors on the surface of the liver cells, thereby decreasing serum LDL level [22]. Recent studies have shown that the efficacy of statins cannot just lipid-lowering effect, it also has the protection of vascular endothelial cells, antioxidant, anti-inflammatory, anti-myocardial remodeling and other effects [23-25]. In our study, ATV could inhibit the proliferation and migration of VSMCs, causing the morphology of VSMCs elongated spindle shape, increasing the expression of SM-MHC and SM-22 $\alpha$ , decreasing the expression of OPN in VSMCs. These results confirmed that ATV could inhibit the phenotypic transformation of VSMC, which may also be one of the mechanisms of anti-atherosclerosis.

Krüppel-like factor (IKLF/KLF-5) was a transcriptional regulator of the KLF family, which was closely related to embryonic development, cell proliferation and tumorigenesis. In addition, various cell lines studies showed that KLF-5 was an important growth promoting gene and differentiation suppressor gene. KLF-5 can promote cell growth and cell cycle progression by accelerating G1/S phase transformation and promoting mitosis in NIH3T3 cells. A study manifested that the expression of KLF-5 was significantly increased in vascular neointima of the VSMCs [26]. Similarly, our findings were consistent with the results of the above studies. Through transfected VSMCs with KLF-5-expressing plasmid, we found that KLF-5 could promote the proliferation and migration

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of VSMCs, and reverse the inhibition effect of ATV on phenotypic transformation of VSMCs.

### Conclusion

In this study, through different concentrations of ATV intervention in VSMCs, ATV has been found to inhibit the phenotype of VSMCs and the expression of KLF-5. In addition, by over-expressing KLF-5 plasmid into VSMCs, it was found that over-expression of KLF-5 could promote the proliferation and migration of VSMCs and reverse the inhibition effect of ATV on phenotypic transformation of VSMCs. Therefore, we can come to the conclusion that ATV inhibited VSMCs phenotype transformation by down regulating KLF-5 in CH rats.

### Disclosure of conflict of interest

None.

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